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Human suppressive neutrophils CD16^{bright}/CD62L^{dim} exhibit decreased adhesion

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ABSTRACT

Neutrophils are essential effector cells in host defense against invading pathogens. Regulation of adhesion, migration, and chemotactic processes is important in the homing and activation of these cells. We recently described three distinct subsets of circulating human neutrophils in peripheral blood during acute systemic inflammation. One subset, CD16^{bright}/CD62L^{dim}, has immune suppressive characteristics because it can inhibit T-cell proliferation. The other two subsets consist of banded CD16^{dim}/CD62L^{bright} and phenotypically mature (normal) CD16^{bright}/CD62L^{bright} neutrophils. The current study was designed to determine the adhesion characteristics of these different neutrophil subsets. Analysis of adhesion to activated endothelium under flow conditions revealed that CD16^{bright}/CD62L^{dim} neutrophils adhered less compared with CD16^{bright}/CD62L^{bright} and CD16^{dim}/CD62L^{bright} neutrophils. This decrease in binding capacity could be mimicked in the other neutrophil subsets by blocking L-selectin. Chemotaxis of CD16^{bright}/CD62L^{dim} neutrophils to the end-target chemoattractant *N*-formylmethionine-leucine-phenylalanine was lower compared with that for the CD16^{dim}/CD62L^{bright} neutrophil subset, whereas chemotaxis to cell-derived chemoattractant CXCL8 was comparable. Our data indicate that capture on endothelium under flow conditions, a key mechanism necessary for extravasation, of CD16^{bright}/CD62L^{dim} neutrophils to inflammatory sites is attenuated, which may facilitate migration of these cells to other tissue localizations. Modulation of this process is a potential target to manipulate inflammation because potentiation of this immune suppression might aid in anti-inflammatory therapy. *J. Leukoc. Biol.* 92: 1011–1020; 2012.

Introduction

Neutrophils are innate immune cells that are essential in the host defense against invading pathogens. These cells respond to signals based on pathogen- and/or damage-associated molecular patterns [1, 2], such as lipopolysaccharide [3] and ATP [2], respectively. Proper orchestration of neutrophil homing during infection and tissue damage is crucial for the outcome of host defense [4]. Upon inflammation, molecules such as E-selectin, ICAM-1, and VCAM-1 are up-regulated on the surface of the endothelial cells. Selectins and their ligands mediate the initial capture under conditions of flow and rolling of neutrophils on the surface of the endothelium [5, 6]. Next, neutrophils are activated by chemokines that are present on the endothelial surface, resulting in inside-out control of their integrins [7]. Both β2-integrins, macrophage antigen 1 (MAC-1; CD11b/CD18) and leukocyte function-associated antigen 1 (LFA-1; CD11a/CD18), on neutrophils are important for firm adhesion and transendothelial migration [5, 6].

Extravasation and migration of neutrophils into the infected or damaged tissues are dependent on sensing of a chemotactic gradient. The chemokines involved in chemotaxis can be divided into two categories. The end-target chemoattractants [e.g., C5a and *N*-formylmethionine-leucine-phenylalanine (fMLF)] are present at the site of the (bacterial) infection and act on many leukocyte subtypes [8]. Cell-derived chemoattractants are more leukocyte type-specific, such as CXCL8 for neutrophils [9]. Leukocytes are able to prioritize signals of the end-target chemoattractants over the signals of cell-derived chemokines [9–11].

Until recently, neutrophils were thought to consist of one population of cells able to phagocytose and kill bacteria at the site of infection followed by rapid apoptosis and clearance by resident macrophages [12]. However, lately neutrophils have also been shown to migrate to and reside in lymph nodes [13,

Abbreviations: fMLF=formylmethionine-leucine-phenylalanine; HUVEC, human umbilical vein endothelial cell; LFA-1=lymphocyte function-associated antigen 1; MAC-1=macrophage antigen 1; MDSC=myeloid derived suppressor cell

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14]. Apart from this traffic into lymphoid tissues, retrograde migration of neutrophils back to the peripheral blood has also been shown in several species [15, 16].

Several studies support the hypothesis that neutrophils are not a homogeneous population [16–18]. We recently showed that acute inflammation evoked by experimental human endotoxemia and trauma is associated with the rapid occurrence of at least three different subsets of neutrophils defined by the expression of CD16 and CD62L [18]: a $CD16^{bright}$/$CD62L^{bright}$, population consisting of phenotypically normal mature neutrophils; a second population showing $CD16^{dim}$/$CD62L^{bright}$ expression and a banded nuclear morphology, characteristic of neutrophils derived from the bone marrow; and a third population of $CD16^{bright}$/$CD62L^{dim}$ neutrophils characterized by a hypersegmented nucleus. These latter cells were able to suppress T-cell proliferation in a MAC-1- and H_2O_2-mediated fashion [18].

Differences in expression of CD62L between the $CD16^{bright}$/$CD62L^{bright}$ and $CD16^{dim}$/$CD62L^{bright}$ neutrophils vs. $CD16^{bright}$/$CD62L^{dim}$ neutrophils may imply differences in adhesion characteristics of the three subpopulations [19]. Therefore, we set out in this study to identify the adhesion characteristics of the different neutrophil phenotypes to gain more insight into mechanisms implicating differential homing of the cells to different tissue locations.

MATERIALS AND METHODS

Subjects and study design

The study protocol was approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre and complies with the Declaration of Helsinki and Good Clinical Practice guidelines. Male volunteers gave written informed consent to participate in the experiments, which were part of larger endotoxin trials (NCT00513110, NCT00783068, NCT00785018, and NCT00916448 at www.clinicaltrials.gov). Subjects were enrolled after screening [20] and prehydrated with 1500 ml of glucose/saline infusion [21]. U.S. reference *Escherichia coli* endotoxin (lot Ec-5; Center for Biologic Evaluation and Research, U.S. Food and Drug Administration, Bethesda, MD) was used in this study at a dose of 2 ng/kg. Ec-5 endotoxin was reconstituted in 5 ml of 0.9% saline and was injected as a single intravenous bolus during 1 min at $t = 0$. Blood samples anticoagulated with sodium heparin were taken from the arterial catheter 3 h after administration of endotoxin.

Reagents

Human serum albumin and pasteurized plasma solution were purchased from the Central Laboratory of the Netherlands Sanquin (Amsterdam, The Netherlands). Recombinant human TNFα was purchased from Roche (Mannheim, Germany). Isolation buffer contained PBS supplemented with pasteurized plasma solution (10%) and trisodium citrate (0.4%, w/v). Incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5% (w/v) HSA. CXCL8 (IL-8) was obtained from PeproTech (London, UK) and C5A from Sigma-Aldrich (St. Louis, MO, USA). fMLF was obtained from Molecular Probes (Leiden, The Netherlands). All other materials were reagent-grade.

Antibodies

Monoclonal antibodies used for flow cytometry were CD11b (clone 2LPM19c) and CD18 (clone MHM23) from DAKO (Heverlee, Belgium); CD16 Alexa 647 (clone 3G8), CXCR1 (clone 5A12), CXCR2 (clone 6C6), CD11c (clone S-HCL-3), CD62L FITC (clone Dreg56), CD62L PE (clone SK11), and CD15 (clone MMA) from Becton Dickinson (San Jose, CA, USA); CD16 FITC (clone LNK16), CD11a FITC (clone 38), and CD88 (clone P12/1) from Serotec (Düsseldorf, Germany; and CD11b (clone cbrm1/5) from BioLegend (San Diego, CA, USA).

Functionally blocking antibodies DREG56 (anti-L-selectin) and IB4 (anti-β_2-integrin) or control antibody W6/32 (IgG1, anti-HLA-A, HLA-B, and HLA-C) were isolated from the supernatant of the hybridoma obtained from American Type Culture Collection (Manassas, VA, USA). Monoclonal antibody NKI-L15 (anti-$\alpha L\beta 2$) was kindly provided by Professor Y. van Kooyk, VUMC, Amsterdam, The Netherlands.

Flow cytometry

Whole blood samples were put on ice, and erythrocytes were lysed in isotonic ice-cold NH_4Cl solution followed by centrifugation at 4°C. Total leukocytes obtained after lyses of erythrocytes were resuspended in isolation buffer and stained with antibodies for 30 min at 4°C. Cells were washed in cold isolation buffer before analysis on FACSCalibur (Becton Dickinson) or sorting. Neutrophil subsets were sorted on FACSAria or FACSVantage, (Becton Dickinson) using CD62L/CD16 as described previously [18].

Endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins according to Jaffe *et al.* [22] with some minor modifications [23]. The cells were cultured in endothelial cell growth medium-2 (BioWhittaker, Walkersville, MD, USA). Cell monolayers were grown to confluence in 5–7 days. Endothelial cells of the second passage or third passage were used in perfusion assays. HUVECs were activated by TNFα (100 U/ml, 4–7 h, 37°C) before the perfusion experiments.

Perfusion chamber

Perfusions under steady flow were performed in a modified form of transparent parallel plate perfusion chamber exactly as described by us previously [23]. This micro chamber had a slit height of 0.2 mm and width of 2 mm. The chamber contained a circular plug on which a coverslip (18 mm × 18 mm) with confluent HUVECs was mounted.

Neutrophil perfusion and evaluation

In vitro flow chamber experiments were performed as described previously [24]. In short, neutrophils were preincubated with control (HB118 directed against HLA A,B,C) or blocking L-selectin (clone Dreg 56) antibodies (10 μg/ml; 450 μl of cells, 1×10^6 cells/ml, 15 min, 37°C) and were aspirated from a reservoir through the perfusion chamber. The neutrophil suspension was perfused during 3 min at shear stress of 1.5 dyn/cm^2 to obtain an endothelial surface with firmly adhering and rolling cells. Thereafter, recording of the images on video was started. To automatically determine the total adhering cells (rolling and firmly adherent), the percentage of rolling cells, and the velocity of rolling cells, custom-made software (Optimas 6.1) was used as described previously [25].

Fluorescent-bead adhesion assay

TransFluor-Spheres (488/645 nm, 1.0 μm; Molecular Probes, Invitrogen, Carlsbad, CA, USA) were covalently coupled to streptavidin using 1-ethyl-3(-3-dimethylaminopropyl)-carbodiimide in MES buffer (pH 6). Then, beads were coated with biotin-SP-AffiniPure goat anti-human Fc(γ) F(ab′)2 (Jackson ImmunoResearch Laboratories, Inc., Suffolk, UK) and subsequently were coated with Fc-ICAM-1. Neutrophils were resuspended in incubation buffer. The ligand-coated beads were washed twice and added together with the cells (40,000/well) and C5a, Mn^{2+}, or anti-β2-integrin blocking monoclonal antibody IB4 (10 μg/ml) in a 96-well V-shaped-bottom plate for 15 min at 37°C. Binding of the fluorescent beads to the neutrophils was determined by flow cytometry using a FACSCalibur, and results

are reported as the percentage of neutrophils positive for ICAM-1-coated beads [26].

Static adhesion assays

Isolated neutrophils were resuspended in incubation buffer at a concentration of 2×10^6 cells/ml. Neutrophils were labeled with calcein for 30 min at 37°C. MaxiSorp plates (96-well; Nalge Nunc International, Rochester, NY) were coated with biotin-SP-AffiniPure goat anti-human Fc(γ) F(ab′)2 (20 μg/ml) in PBS (1 h, room temperature), washed twice with PBS, and subsequently coated with Fc-ICAM-1 (10 μg/ml) (1 h, room temperature). Plates were washed twice with PBS and incubated with incubation buffer until use. Calcein-labeled neutrophils were allowed to adhere to coated plates for 15 min at 37°C in the presence of control or integrin blocking antibodies before stimuli were added. After addition of the stimuli, cells were incubated for another 30 min at 37°C. Total input of cells was measured in a fluoro-luminometer (FLUOstar OPTIMA, BMG LABTECH, Ortenberg, Germany). Nonadherent cells were removed by 4 washing steps, and plates were measured again. Adhesion is depicted as % adhesion of input. Adhesion blocked with anti-CD11b (clone 44a) was defined as background and subtracted from the adhesion with anti-HLA-1 (clone W6/32) control antibody.

Migration assay using the Boyden chamber

Neutrophil migration was measured in the modified Boyden chamber assay as described previously [27]. In short, cellulose nitrate filters (pore width 8 μm, thickness 150 μm; Sartorius AG, Goettingen, Germany) were soaked in incubation buffer, and neutrophils (25 μl of 1×10^6/ml) were added per well and allowed to migrate for 1.5 h at 37°C. Filters were fixed, stained with hematoxylin (Weigert's method), and embedded in malinol. Filters were analyzed by an image analysis system (Quantimet 570C; Leica, Wetzlar, Germany) and an automated microscope to score the number of cells at 15 intervals of 10 μm in the z-direction of the filters. The results are expressed as the chemotactic index, indicating the mean migrated distance (in micrometers), excluding cells with migration 0.

Statistical analysis

Every n depicted in the graphs stands for a different endotoxin volunteer and therefore also for the number of times the experiment has been repeated. Data were analyzed using GraphPad Prism 4 or SPSS 20. Results are expressed as means ± SD. Between-group differences were assessed via one-way ANOVA with a Tukey posttest. A value of $P < 0.05$ was considered to be significant. For analyses of the dose-response curves with their correlated data, a linear mixed model for repeated measurements was used. Fixed factors in the model were concentration and type of neutrophil and the interaction between concentration and type of neutrophil. The latter interaction indicates whether the differences among the types of neutrophil responses were concentration-dependent. Graphically the latter translates into diverging dose-response curves. The fit of the model was assessed via the restricted −2 log likelihood. Post hoc tests were done using a least significant difference approach under the protection of significant effects of the main linear mixed-model analysis.

RESULTS

Expression of adhesion molecules on different subsets of neutrophils after endotoxin challenge

Under nonpathological homeostatic conditions, neutrophils are present as a homogeneous population of leukocytes in the peripheral blood. However, we have recently reported that distinct populations of peripheral neutrophils were present in peripheral blood during systemic inflammation: CD16^{dim}/CD62L^{high}, CD16^{high}/CD62L^{high}, and CD1^{high}/CD62L^{dim}. This last subset is able to suppress T-cell proliferation [18].The differences in phenotype and function among the neutrophil populations led us to hypothesize that they might also differ in adhesion and migration characteristics.

To investigate this hypothesis, we first tested the neutrophil populations on expression of molecules that are associated with adhesion and migration. To isolate the different neutrophil phenotypes, we evoked acute systemic inflammation in healthy volunteers by injection of endotoxin (2 ng/kg) [18]. Neutrophil subsets were analyzed in blood samples taken 3 h after endotoxin administration because the percentage of CD16^{bright}/CD62L^{dim} neutrophils peaked at this time point [18]. Expression of adhesion receptors was measured by triple staining using a flow cytometer. The different neutrophil subsets were identified by first gating all granulocytes according to their forward/sideward scatter characteristics followed by analysis of differential expression of FcγRIII (CD16) and L-selectin (CD62L).

CD16^{bright}/CD62L^{dim} neutrophils were characterized by low expression of L-selectin (CD62L) (**Fig. 1A**). However, no differences were detected in the expression of Lewis X antigens (CD15) that are present on selectin ligands (Fig. 1B). The integrin expression of CD11c (αX) on CD16^{bright}/CD62L^{dim} neutrophils was remarkably increased compared with that for both other subsets (Fig. 1C). Likewise, CD18, the common β2-integrin chain, and CD11b, the αM-integrin chain, were higher on the CD16^{bright}/CD62L^{dim} neutrophil subset than in the banded cells (Fig. 1C). The only integrin tested that did not show differences in expression between subsets was CD11a, the α-chain of LFA-1 (Fig. 1C). In addition, the expression of the activation epitope of CD11b/CD18 determined by antibody CBRM1/5 was higher in the CD16^{bright}/CD62L^{dim} subset than in the other two subsets of neutrophils (Fig. 1D). CBRM1/5 expression was unimodal, so expression was increased on all CD16^{bright}/CD62L^{dim} neutrophils. Stimulation with Mn^{2+} or fMLF did not increase this expression of CBRM1/5 on the CD16^{bright}/CD62L^{dim} subset (data not shown). CD49d, the α-chain of VLA-4, was not detected on any of the subsets (data not shown).

Rolling and adhesion characteristics under flow conditions

Because these differences in receptor expression among the neutrophil subsets were likely to affect the adhesion characteristics of the different neutrophil phenotypes, we tested their adhesion under flow conditions to activated endothelial cells [28]. Cells were perfused for 3 min in an in vitro flow chamber over a confluent layer of TNFα-activated (10 U/ml, 4–8 h, 37°C) HUVECs. The number of cells captured on the endothelium and the percentage of rolling cells were determined off-line by image analysis [29]. Capture was defined as the number of neutrophils per mm^2 in contact with the endothelium after a fixed perfusion time. This analysis did not discriminate between rolling and firmly adhering cells. Despite their activated phenotype (Fig. 1), the capture of CD16^{bright}/CD62L^{dim} cells on the endothelium was significantly lower than that found for the CD16^{bright}/CD62L^{bright} and CD16^{dim}/CD62L^{bright} subsets (**Fig. 2A**). The CD16^{bright}/CD62L^{dim} neu-

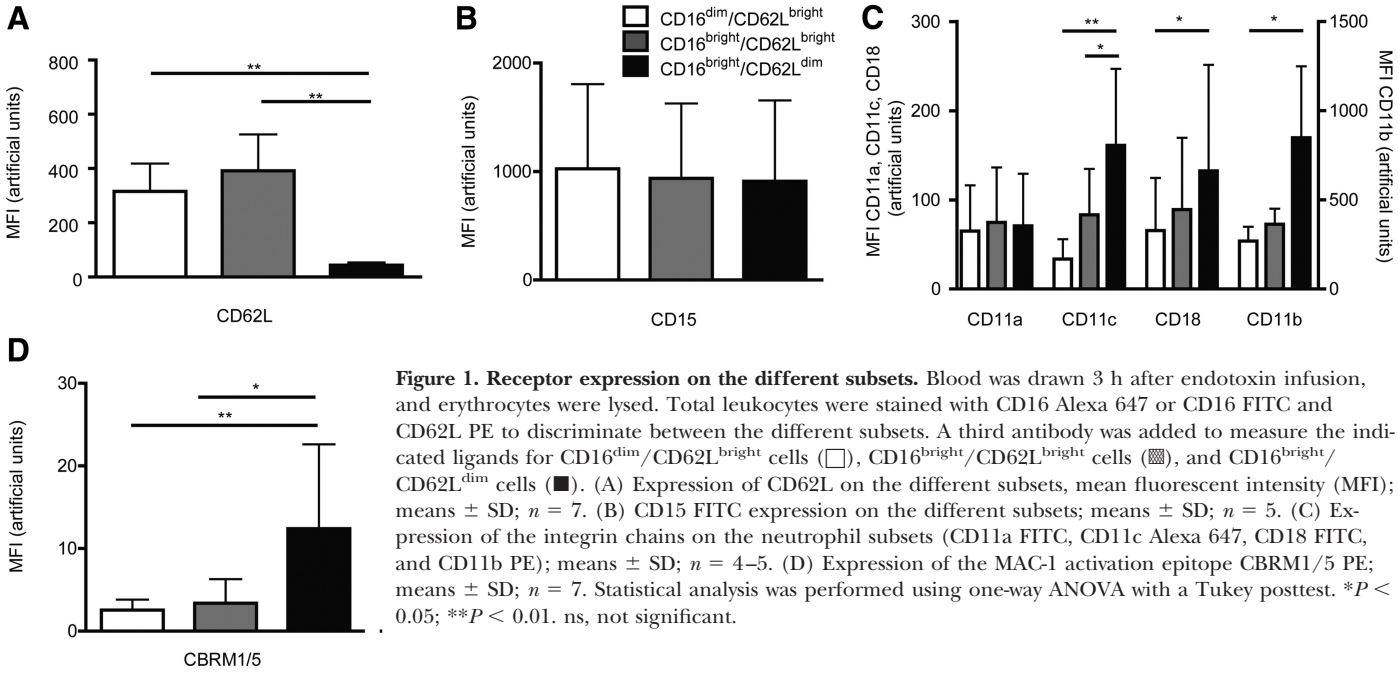

Figure 1. Receptor expression on the different subsets. Blood was drawn 3 h after endotoxin infusion, and erythrocytes were lysed. Total leukocytes were stained with CD16 Alexa 647 or CD16 FITC and CD62L PE to discriminate between the different subsets. A third antibody was added to measure the indicated ligands for $CD16^{dim}/CD62L^{bright}$ cells (□), $CD16^{bright}/CD62L^{bright}$ cells (▨), and $CD16^{bright}/CD62L^{dim}$ cells (■). (A) Expression of CD62L on the different subsets, mean fluorescent intensity (MFI); means ± SD; $n = 7$. (B) CD15 FITC expression on the different subsets; means ± SD; $n = 5$. (C) Expression of the integrin chains on the neutrophil subsets (CD11a FITC, CD11c Alexa 647, CD18 FITC, and CD11b PE); means ± SD; $n = 4$–5. (D) Expression of the MAC-1 activation epitope CBRM1/5 PE; means ± SD; $n = 7$. Statistical analysis was performed using one-way ANOVA with a Tukey posttest. $*P < 0.05$; $**P < 0.01$. ns, not significant.

trophils had a capture of 215 ± 52 cells/mm^2 compared with 282 ± 71 and 278 ± 38 cells/mm^2 ($P<0.01$) for the $CD16^{dim}/CD62L^{bright}$ and $CD16^{bright}/CD62L^{bright}$ neutrophils, respectively, a decrease in capture of more than 20%. This could not be explained by enhanced detachment of $CD16^{bright}/CD62L^{dim}$ neutrophils from the surface because careful analysis of the video streams showed no difference in numbers of detaching cells (results not shown). This result indicates that the initial capture and not the detachment of the $CD16^{bright}/CD62L^{dim}$ neutrophils is lower, resulting in fewer cells on the surface. Despite the differences in CD11b, CD18, and CBRM1/5 expression, the ratio between adherent cells and rolling cells was not significantly different (Fig. 2B). Because the expression of CD11a did not differ among subsets, the relative contribution of both MAC-1 and anti-LFA-1 in our rolling system was tested using blocking antibodies. Blockage of only one of the integrins was not sufficient to significantly decrease the amount of adhering cells; the combination of anti-LFA-1 and anti-MAC-1 was required (Supplemental Fig. 1).

L-selectin has been described to be important for capture of neutrophils under flow conditions [19, 30]. To investigate whether the low expression of L-selectin observed in $CD16^{bright}/CD62L^{dim}$ neutrophils was related to their decreased adhesion, L-selectin was blocked in the different neutrophil subsets by a blocking antibody (DREG56). Blocking of L-selectin resulted in significantly reduced capture of $CD16^{dim}/CD62L^{bright}$ and $CD16^{bright}/CD62L^{bright}$ neutrophils but had no significant effect on the capture of $CD16^{bright}/CD62L^{dim}$ neutrophils (Fig. 2C). Furthermore, blocking L-selectin abrogated the differences in capture among the subsets (Fig. 2C). This finding demonstrates that L-selectin is indeed important for neutrophil capture on activated endothelium and that this is the likely cause of the observed decrease in capture of $CD16^{bright}/CD62L^{dim}$ neutrophils.

L-selectin has been described to regulate neutrophil capture either by primary or by secondary tethering [19] . Secondary tethering would lead to clustering of cells on the endothelial surface characterized by an increased cluster index[28, 30]. To investigate whether the difference in adhesion among the subsets was caused by secondary tethering, we calculated the cluster index. There were no differences in the cluster index of the neutrophil subsets (Fig. 2D). Moreover blocking of L-selectin did not influence the cluster index (Fig. 2D). This finding indicates that primary rather than secondary tethering plays a role in this experimental setup. In addition, the median velocity of the rolling cells did not differ among the neutrophil subsets; L-selectin blockage did not influence the velocity (Fig. 2E).

$CD16^{bright}/CD62L^{dim}$ and $CD16^{bright}/CD62L^{bright}$ neutrophils are characterized by increased MAC-1-dependent static binding; LFA-1-dependent binding is similar among subsets

We next determined the contribution of individual integrins to the binding to vascular adhesion molecules for the different neutrophil subsets. For this, adhesion of the β2-integrins to their main ligand ICAM-1 (CD54) was tested in two different adhesion assays.

The first assay applied ICAM-1-coated beads; the binding of neutrophils to these beads was shown to be LFA-1 (and not MAC-1)-mediated as only blocking antibodies directed against LFA-1 blocked this response (results not shown). The three neutrophil subsets showed similar percentages of binding to ICAM beads, between 7 and 9% (**Fig. 3A**), indicating that not only the expression (Fig. 1C) but also the activity of LFA-1 was similar in all phenotypes.

The second assay measures neutrophil binding on ICAM-coated plates. This assay is completely MAC-1-dependent because blocking antibodies completely blocked this response

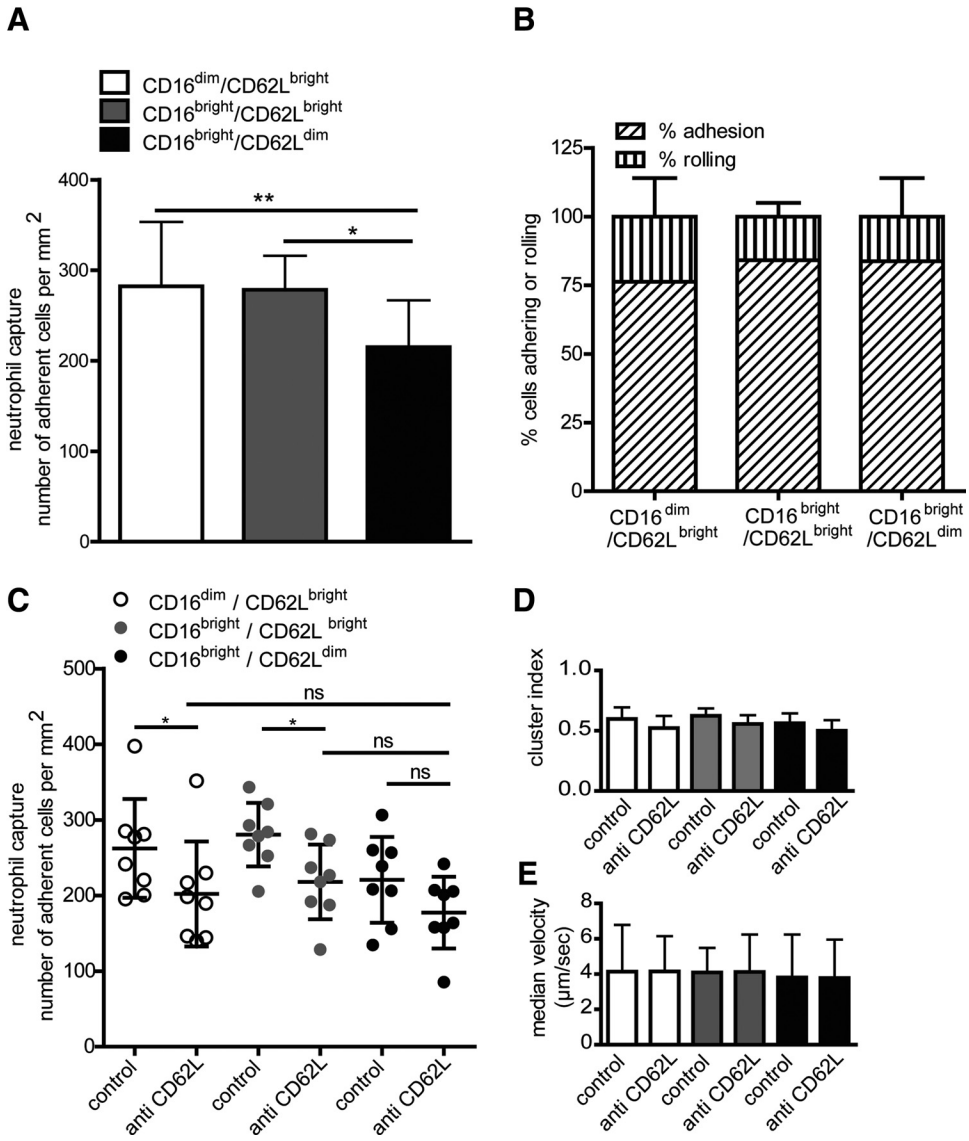

Figure 2. Rolling and adhesion under flow. Neutrophil subsets were sorted, and cells were perfused over TNF-α activated HUVECs. (A) Capture of neutrophils per mm^2 endothelium was determined; mean ± SD; $n = 10$. (B) Percentage rolling cells vs. percentage adhering cells was determined; means ± SD; $n = 9$. (C–E) Neutrophils were preincubated with control antibody anti-HLA (W6/32) or anti-CD62L (DREG56) 15 min before perfusion. (C) Capture of neutrophils per mm^2 endothelium; means ± SD; $n = 8$. (D) Cluster index of neutrophils perfused over activated endothelium; means ± SD; $n = 8$. (E) Median velocity (μm/s) of the rolling neutrophils; means ± SD; $n = 9$. Statistical analysis was performed using one-way ANOVA with a Tukey posttest. $^*P < 0.05$; $^{**}P < 0.01$.

(Supplemental Fig. 2). The MAC-1-dependent adhesion of CD16^{bright}/CD62L^{bright} and CD16^{bright}/CD62L^{dim} neutrophils was similar; both subsets adhered more than CD16^{dim}/CD62L^{bright} neutrophils (Fig. 3B). The differences in binding can, however, not be explained by the expression patterns of CD11b because both the total (clone 2LPM19c) (Fig. 1C) and activated (clone CBRM1/5) (Fig. 1D) Mac-1 was highly expressed on CD16^{bright}/CD62L^{dim} neutrophils but not on the CD16^{bright}/CD62L^{bright} and CD16^{dim}/CD62L^{bright} neutrophils (Fig. 1C and D). Numbers in this graph are corrected for background binding using MAC-1 blocking antibody (clone 44a); slightly higher binding after antibody addition

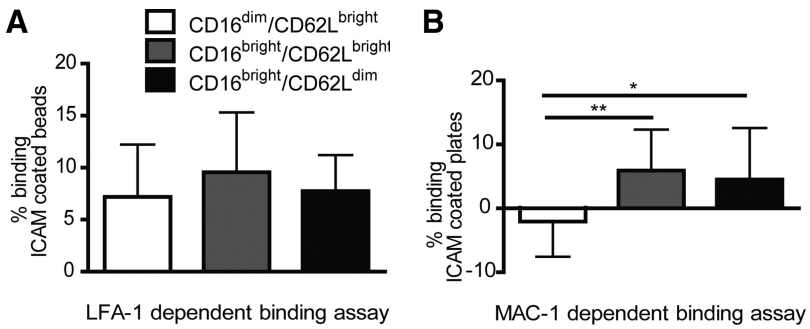

Figure 3. MAC-1- and LFA-1-dependent ICAM-1 binding. Neutrophils subsets were sorted. (A) Cells were incubated for 30 min at 37°C with ICAM-coated beads with or without CD18 blocking antibody. After 30 min, samples were put on ice and analyzed by flow cytometry. Background binding (determined using anti-CD18) was subtracted. Data are expressed as mean ± SD; $n = 11$. (B) Cells were stained with calcein and incubated for 45 min at 37°C on ICAM-coated plates. Total fluorescence was measured before washing and after 4 washes to calculate the percentage adherence. Background binding (determined using anti-CD11b antibody) was subtracted. Data are expressed as mean ± SD; $n = 7$. Statistical analysis was performed using one-way ANOVA with a Tukey posttest. $^*P < 0.05$; $^{**}P < 0.01$.

resulted in negative values for CD16^{dim}/CD62L^{bright} neutrophils.

Differences in chemotactic response of neutrophils subsets toward end-target chemoattractant fMLF

Next, migration of neutrophils toward end-target chemoattractant fMLF and the cell-derived chemoattractant CXCL8 was induced. These chemotaxins induced chemotaxis in all subsets, but the percentage of cells responding to the chemotaxins did not differ among subsets (data not shown). However, the migration distance of the CD16^{dim}/CD62L^{bright} subset toward fMLF (10^{-8} M) was significantly increased (51±15 μm) compared with the migration of the CD16^{bright}/CD62L^{dim} neutrophil subset (38±12 μm) (**Fig. 4A**). The migration of the CD16^{bright}/CD62L^{bright} subset was intermediate (47±15 μm) and not significantly different from that of CD16^{bright}/CD62L^{dim} or CD16^{dim}/CD62L^{bright} neutrophils. In contrast, the chemotaxis toward IL-8 (10^{-8} M) was comparable among all three subsets (Fig. 4A). Differences in chemotaxis could not be explained by differences in receptor expression. CXCL8 receptor CXCR2 (CD182) had lower expression on the CD16^{dim}/CD62L^{bright} cells compared with expression for the other two subsets (Fig. 4B). These differences among neutrophil subsets did not, however, result in altered chemotaxis toward CXCL8. The expression of the other CXCL8 receptor, CXCR1 (CD181), did not differ (Fig. 4B).

Chemoattractants are known for their bell-shaped dose-response curves. To test whether a shift in this bell-shaped curve was responsible for the decreased chemotaxis of CD16^{bright}/CD62L^{dim} neutrophils, dose-response curves for fMLF were performed. The fMLF concentration curves of the CD16^{bright}/CD62L^{dim} and CD16^{bright}/CD62L^{bright} neutrophils were significantly lower over the whole range of the curve than those for the CD16^{dim}/CD62L^{bright} neutrophils (P=0.013 and P=0.018, respectively) (Fig. 4C). In addition, the concentration curves of C5a, another end-target chemoattractant, were measured. These did not show clear differences in chemotaxis of the different neutrophils (Fig. 4D), suggesting similar sensitivity for C5a of the different phenotypes. Receptor expression of the C5a receptor CD88 did not differ among subsets (Fig. 4E).

CD16^{bright}/CD62L^{dim} neutrophils show increased MAC-1-dependent binding after CXCL8 and fMLF activation

Chemotaxis, in particular toward fMLF, differed among neutrophil subsets. This difference could be due to an altered response upon fMLF stimulation. Functionality upon fMLF (10^{-8} M), C5a (10^{-8} M), or CXCL8 (10^{-8} M) stimulation was tested in integrin-specific adhesion assays. LFA-1-dependent adhesion increased after stimulation with both fMLF and C5a (**Fig. 5A**). There was no difference in fMLF sensitivity among the subsets. The CD16^{bright}/CD62L^{dim} subset was, however,

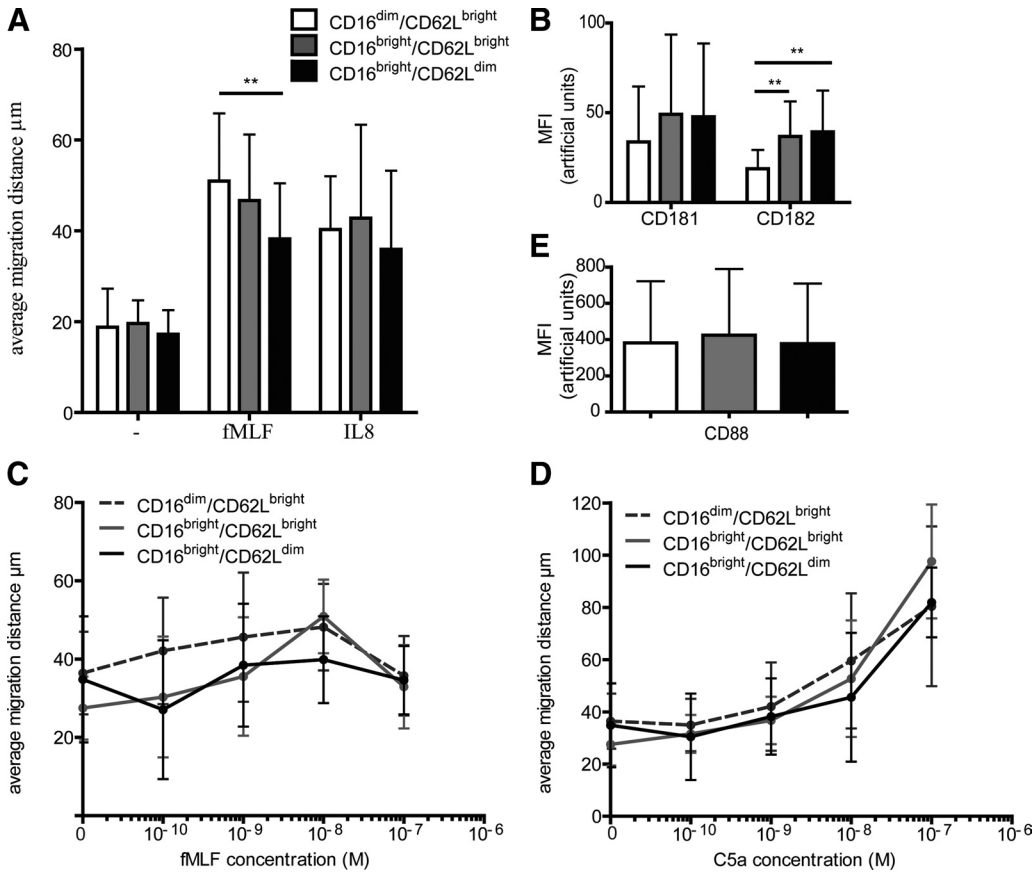

Figure 4. Chemotaxis and chemokine receptor expression. (A) Neutrophil subsets were sorted and put in a Boyden chamber and allowed to migrate toward the chemoattractant gradient for 45 min. Afterwards filters were stained and average migration distance was calculated; means ± SD; n = 6–13. (B) Blood was drawn 3 h after endotoxin infusion and erythrocytes were lysed. Total leukocytes were stained with CD16 and CD62L to discriminate between subsets. A third antibody against the CXCR1 (CD181) and CXCR2 (CD182) receptors was added and samples were measured by flow cytometry, means ± SD; n = 4–5. (C and D) Chemotaxis to a dose-response curve of fMLF (C) and C5a (D) was tested using Boyden chambers. (E) Blood was drawn 3 h after endotoxin infusion, and erythrocytes were lysed. Total leukocytes were stained with CD16 and CD62L to discriminate between subsets. A third antibody against the C5a receptor (CD88) was added, and samples were measured by flow cytometry; means ± SD; n = 4–5. Statistical analysis was performed using one-way ANOVA with a Tukey posttest; for the dose-response curves a mixed model for repeated measurements was used (see Materials and Methods). *$P < 0.05$; **$P < 0.01$.

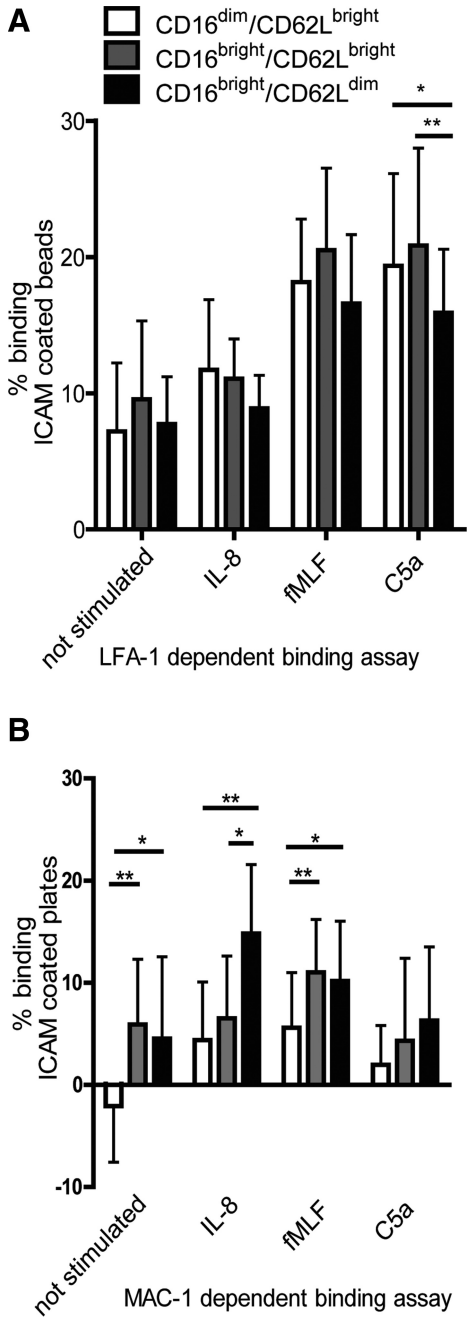

Figure 5. LFA-1- and MAC-1-dependent ICAM-1 adhesion after activation. Neutrophils subsets were sorted. (A) Cells were incubated for 30 min at 37°C with ICAM coated beads with or without CD18 blocking antibody and/or stimuli (CXCL8, 10^{-8} M; C5a, 10^{-8} M; fMLF, 10^{-8} M) After 30 min, samples were put on ice and analyzed by flow cytometry. Background binding (determined using anti-CD18) was subtracted. Data are expressed as means ± SD; $n = 6$–11. (B) Cells were stained with calcein and incubated 15 min at 37°C in ICAM-coated plates with control antibody anti-HLA (W6/32) or anti-CD11b (44a) before stimulation. Stimuli (CXCL8, 10^{-8} M; C5a, 10^{-8} M; fMLF, 10^{-8} M) were added after 15 min, and cells were incubated for another 30 min at 37°C. Total fluorescence was measured before washing and after 4 washes to calculate the percentage adherence. Background binding (determined using anti-CD11b antibody) was subtracted. Data are expressed as means ± SD; $n = 7$. Statistical analysis was performed using one-way ANOVA with a Tukey posttest. *$P < 0.05$; **$P < 0.01$.

less sensitive for C5a-induced LFA-1 adhesion (Fig. 5A). Adhesion in the MAC-1-dependent assay increased upon CXCL8 and fMLF stimulation (Fig. 5B). The enhanced adhesion of the CD16^{bright}/CD62L^{bright} and CD16^{bright}/CD62L^{dim} neutrophils compared with that of the CD16^{dim}/CD62L^{bright} neutrophils was still present after activation (Fig. 5A and B). Numbers in this graph are corrected for background binding using MAC-1-blocking antibody (clone 44a); slightly higher binding after antibody addition resulted in negative values for CD16^{dim}/CD62L^{bright} neutrophils in the unstimulated situation.

DISCUSSION

The present study demonstrates that the different neutrophil subsets found during acute systemic inflammation are characterized by differences in adhesion and migration characteristics (**Fig. 6**). CD16^{bright}/CD62L^{dim} neutrophils showed higher expression of CD18, CD11c, and active CD11b. However, these CD16^{bright}/CD62L^{dim} neutrophils showed diminished capture on activated endothelium of more than 20% under flow conditions compared with CD16^{bright}/CD62L^{bright} and CD16^{dim}/CD62L^{bright} neutrophils due to low expression of L-selectin. It is tempting to speculate that the CD16^{bright}/CD62L^{dim} neutrophils are less capable of extravasation to inflamed tissue because of their decreased capture to activated endothelium. Furthermore, the chemotaxis to end-target chemoattractant fMLF but not to cell-derived chemoattractant CXCL8 was impaired in CD16^{bright}/CD62L^{dim} neutrophils compared with that in CD16^{dim}/CD62L^{bright} neutrophils but not compared with that in CD16^{bright}/CD62L^{bright} neutrophils.

The diminished capture of the CD16^{bright}/CD62L^{dim} subset of neutrophils to activated endothelium could be explained, at least in part, by the low expression of L-selectin. The 20% difference in capture was relatively small. However, the difference was persistent even after cell sorting. In addition, the 20% reduction is a substantial difference, considering the big redun-

		CD16^{dim}/CD62L^{bright}	CD16^{bright}/CD62L^{bright}	CD16^{bright}/CD62L^{dim}
Capture on endothelium		+++	+++	++
chemotaxis	IL8	++	++	++
	fMLF	++	+	+
	C5a	+++	+++	+++
LFA-1 binding	IL8	+	+	+
	fMLF	+++	+++	+++
	C5a	+++	+++	++
MAC-1 binding	IL8	+	+	+++
	fMLF	++	+++	+++
	C5a	+	+	+

Figure 6. Conclusion and overview of experimental results. A schematic overview of the results shown in Figs. 2–5 is presented.

dancy in migratory molecules. This decreased L-selectin expression is probably contributing to the decreased adhesion capacity because inhibition of L-selectin function on $CD62L^{bright}$ cells with a blocking antibody affected their adhesion characteristics similarly. Because $CD16^{bright}/CD62L^{dim}$ neutrophils still express CD62L, although to a lesser extent than the other neutrophil subsets, a decrease in capture upon CD62L blockage was to be expected. Indeed, adhesion of $CD16^{bright}/CD62L^{dim}$ neutrophils to endothelial cells was found to be sensitive to blockade of CD62L. Previous in vitro studies had demonstrated that L-selectin is important for leukocyte capture on activated endothelium under flow conditions. There are at least two mechanisms by which L-selectin can mediate capture to activated endothelium: primary and secondary tethering [30]. In humans, L-selectin can mediate primary capture by binding to E-selectin on the activated endothelium [31]. Secondary capture (secondary tethering) refers to the process in which already adherent leukocytes facilitate the capture of other leukocytes [19, 30]. In mice, a significant role of L-selectin in adhesion to endothelial cells has been described in larger venules and arterial vessels [30]. To determine whether L-selectin was important for secondary capture of neutrophils in our flow system, the cluster index was analyzed [19]. We found that the cluster index was low and not significantly different among the neutrophil subsets, indicating that the effects on adhesion in this in vitro system are due to primary capture and not secondary capture.

Apart from selectins, integrins play an important role in leukocyte recruitment [32]. The $CD16^{bright}/CD62L^{dim}$ neutrophils showed enhanced expression of integrins CD18, CD11c, and active CD11b (clone CBRM1/5). The clear difference in expression of active CD11b could not be directly translated to functionality evaluated in our MAC-1-dependent adhesion seen in the ICAM binding assay. It appears that avidity rather than affinity of integrins is important for adhesion of neutrophils [33]. Indeed, blockade of protein kinase Cζ can induce a significant increase in CBRM1/5 expression on human neutrophils without induction of integrin clustering [34]. Apparently, this increase in expression was not enough to increase adhesion of the neutrophils. Integrin avidity rather than increased affinity proved to play an important role in MAC-1 adhesion [34]. Our data are consistent with this hypothesis; the high expression of CBRM1/5, indicative of enhanced affinity, was not sufficient to increase the number of adhering cells on ICAM-coated plates.

The increased adhesion of $CD16^{bright}/CD62L^{bright}$ and $CD16^{bright}/CD62L^{dim}$ neutrophils in the static MAC-1-binding assay was not related to enhanced transition from rolling to adherent cells on endothelium in our adhesion assays under flow conditions. No significant differences in the ratio between rolling and adherent cells were detected. The underlying mechanism is not known, but parallel redundant pathways mediated by LFA-1 (Supplemental Fig. 1) might compensate for MAC-1-dependent adhesion differences in our flow system. We have shown that T-cell suppression is mediated via MAC-1 [18]. MAC-1 expression could be up-regulated to increase the efficiency of T-cell suppression, whereas adhesion was not influenced, probably because of the complex interplay between receptor affinity and avidity. The differences in CD62L-dependent adhesion characteristics among different phenotypes raise the question of which consequences these may have in vivo. This is particularly important for the immune $CD16^{bright}/CD62L^{dim}$ phenotype because it would need to be in the proximity of T cells to exhibit its function. The relevance of such immune modulation is acknowledged in a similar suppressive neutrophil phenotype described in particular in mice: myeloid derived suppressor cells (MDSCs). Although of different origin (young neutrophils/monocytes), MDSCs also exhibit several mechanisms for T-cell suppression. These MDSCs are reported to accumulate in the peripheral lymphoid organs, such as the spleen, under conditions of systemic inflammation [35, 36]. It is known from L-selectin knockout mice that T-cell homing to the spleen can be L-selectin-independent [37, 38], and in humans a subset of L-selectin-negative T cells was identified in the spleen [39]. Therefore, it is tempting to speculate that the $CD16^{bright}/CD62L^{dim}$ neutrophils preferentially migrate toward the spleen and are able to suppress splenic T-cell responses.

After adhesion to the endothelium and transendothelial migration, neutrophils migrate to the tissues via chemotaxis. $CD16^{dim}/CD62L^{bright}$ neutrophil chemotaxis toward end-target chemoattractant fMLF was increased over the whole concentration curve compared with that for the other subsets. In contrast, chemotaxis to the cell-derived chemotaxin CXCL8 (10^{-8} M) was similar among neutrophil subsets. This differs from the migration characteristics of neutrophilic MDSCs found in human cancer, which show decreased chemotactic capacity toward CXCL8 [40]. This study clearly describes a different subset of suppressive neutrophils because this subset has lower expression of CXCR1 and CXCR2 compared with that for control neutrophils, and we find similar expressions. They also show up-regulation of CXCR4 on the MDSCs, whereas our cells express only low amounts of CXCR4 comparable to those for control neutrophils (data not shown).

A dose-response curve of fMLF and C5a was used to test whether there was a shift in sensitivity for end-target chemoattractants. The response curve from C5a did not show significant differences among the neutrophil subsets. Chemotaxis toward fMLF, however, was lower in both the $CD16^{bright}/CD62L^{bright}$ and $CD16^{bright}/CD62L^{dim}$ neutrophil subset. Sensitivity for fMLF chemotaxis did not shift; it was lower over the complete concentration curve. It is known that in vitro neutrophil priming with TNFα leads to decreased chemotaxis toward C5a and fMLF [41, 42]. Because experimental human endotoxemia results in markedly elevated TNFα levels in blood [43], neutrophils could have been primed in vivo by cytokines, leading to reduced chemotaxis. The concentration curves show that C5a is a stronger chemoattractant; therefore, C5a might be less sensitive for this priming. In addition, in eosinophils, chemotaxis toward fMLF is sensitive for priming, whereas C5a-mediated chemotaxis is not [44, 45].

In conclusion, $CD16^{dim}/CD62L^{bright}$ neutrophils show decreased capture on activated endothelium under flow conditions. They also migrate less toward end-target chemoattractant fMLF (10^{-8} M), whereas $CD16^{bright}/CD62L^{dim}$ neutrophils show a low migration phenotype. The $CD16^{bright}/$

CD62L^{bright} neutrophils display intermediate migration compared with that for the other subsets. Knowledge of different neutrophil subsets, their function, and their migration characteristics could lead to a better understanding of acute and chronic inflammation. Neutrophils are known to cause tissue damage due to antimicrobial peptides and ROS production. In diseases such as rheumatoid arthritis, chronic obstructive pulmonary disease, and cystic fibrosis neutrophils mediate tissue destruction [46–48]. Part of the neutrophil recruitment in these diseases is thought to be regulated by Th-17 T cells [46, 47, 49]. Therefore, it would be interesting to see whether CD16^{bright}/CD62L^{dim} neutrophils are involved in the disease processes. Anti-L-selectin therapy might repress neutrophil influx in the inflammatory tissue, whereas CD16^{bright}/CD62L^{dim} neutrophils are still able to suppress T-cell proliferation in lymphoid organs. Targeting the neutrophil population as a whole will affect both cytotoxic and immune modulatory functions of neutrophils, possibly leading to unanticipated complications. Increased knowledge of neutrophil subsets will elucidate potential intervention targets, allowing specific targeting of these subsets.

AUTHORSHIP

V.M.K. performed experiments, analyzed data, and wrote the manuscript, with the contribution of J.P. and L.H.U. P.P. supervised the endotoxin challenge experiments. L.H.U. and L.K. designed and coordinated the study. All authors discussed the results and commented on the manuscript.

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